

The cytochrome P450 gene family CYP157 does not contain EXXR in the K-helix reducing the absolute conserved P450 residues to a single cysteine

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Abstract In this work, we have spectroscopically characterised CYP157C1 from *Streptomyces coelicolor* A3(2) which has the motif E²⁹⁷QSLW³⁰¹ rather than the invariant EXXR motif in the P450 K-helix. Site-directed mutagenesis of native E²⁹⁷QSLW³⁰¹ in CYP157C1 to E²⁹⁷ESLR³⁰¹ or E²⁹⁷QSRW³⁰¹ both containing standard EXXR motifs produced cytochrome P420 proteins thought to be inactive forms of P450 even though wild type CYP157C1 has the spectral properties of a normal P450. These results indicate that the EXXR motif is not required in all CYP tertiary architectures and only a single cysteine residue, which coordinates as the fifth thiolate ligand to the P450 haem iron, is invariant in all CYPs structures.

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1. Introduction

Cytochromes P450 (CYP) represent a superfamily of haem-containing enzymes that catalyse the monooxygenation of a wide array of structurally diverse substrates [1,2]. Genome sequencing projects have revealed the enormous biodiversity of the superfamily with >6500 CYPs being identified in bacteria, fungi, insects, worms, plants, fish and mammals (<http://drnelson.utmem.edu/CytochromeP450.html>; <http://p450.antibes.inra.fr>; <http://Arabidopsis-P450.biotec.uiuc.edu>). Catalytically, upon substrate binding, CYPs are initially reduced by a single electron transfer from an ancillary protein(s), which is generally, ferredoxin (fd) and ferredoxin reductase (fpr) for prokaryotic CYPs, NADPH cytochrome P450 reductase (CPR) for eukaryotic microsomal CYPs and adrenodoxin and adrenodoxin reductase for eukaryotic mitochondrial CYPs, followed by oxygen binding and further reduction by a second electron that then allows CYP-catalysed molecular

scission of oxygen to proceed. Finally, one atom of oxygen is inserted into the substrate while the second atom is reduced to water via two protons from the bulk solvent interface (for reviews see [3–5]).

Previously, continued analysis of all CYP amino acid sequences deposited in a variety of databases revealed only three residues which were thought to be absolutely conserved in CYP proteins. The first residue is an invariant cysteine which constitutes the fifth ligand to the haem within the Cys pocket adjacent to the L-helix [6]. This cysteine residue is part of a stretch of nine amino acids forming a β bulge region in which three residues, two glycines and one phenylalanine are generally, but not absolutely, conserved. The second and third invariant residues in most P450s are glutamic acid and arginine within the conserved EXXR motif found in the K-helix [7]. Both of these residues and either a histidine, arginine or asparagine from the meander region form a set of salt-bridge interactions that participate in the formation of the final CYP tertiary structure with increasing evidence suggesting that EXXR is linked somehow to haem association with the CYP polypeptide ([8], Fig. 1). In support of this notion, site-directed mutagenesis of the invariant cysteine, glutamic acid or arginine in individual CYPs resulted in the formation of completely inactive and misfolded P450 isoforms [9,10].

Here, we report a novel CYP subfamily from *Streptomyces* sp. (CYP157C) [11] in which most members lack the EXXR motif previously thought to be invariant. We show that CYP157C1, which contains Q²⁹⁸XXW³⁰¹ instead of EXXR (Fig. 2), is indeed a cytochrome P450 and that site-directed mutagenesis of this K-helix region to a variety of other amino acids including the EXXR motif results in improperly folded P420 protein. Our results demonstrate that the EXXR region is not as essential to CYP architecture as previously thought and that the only invariant residue in all CYP proteins should now be considered to be the cysteine that is the fifth ligand with the haem iron.

2. Materials and methods

2.1. General methods

Reduced carbon monoxide difference spectra for quantification of cytochrome P450 content were measured and calculated according to the method described by Omura and Sato [12]. Protein quantifications

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Abbreviations: CYP, cytochrome P450; PCR, polymerase chain reaction

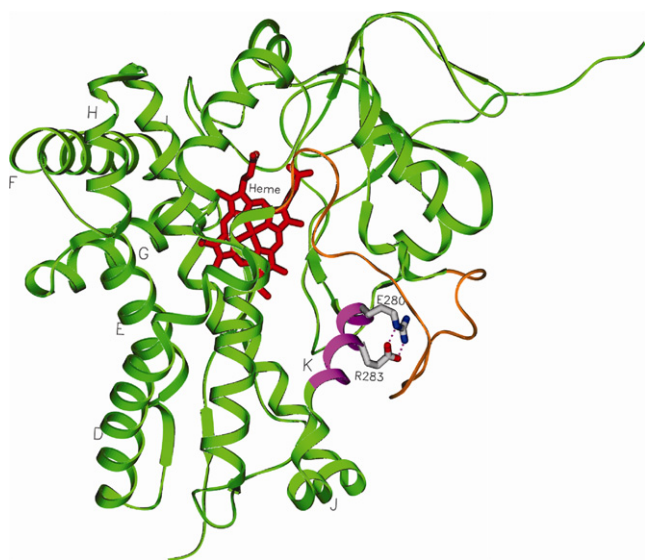


Fig. 1. View of the essentially invariant EXXR motif in the crystal structure of CYP158A2. Glutamic acid and arginine residues (shown with grey/blue and grey/red side chains, respectively), part of the EXXR motif in the K-helix (shown with a purple backbone) and either a histidine, arginine or asparagine from the meander region (shown with a gold backbone) form a set of salt-bridge interactions that are proposed to participate in the formation of the final CYP tertiary structure in order to facilitate haem incorporation (shown in red).

were performed using the bicinchoninic acid assay using bovine serum albumin as a standard. Unless otherwise stated, all chemicals were supplied by Sigma Chemical Company (Poole, Dorset, UK).

2.2. Expression and mutagenesis

The CYP157C1 protein was genetically engineered to contain a His₄-tag at its carboxy-terminus using a polymer chain reaction (PCR)-based strategy as described previously [11]. The resulting CYP157C1WT-His₄ sequence was then cloned into pET17b (yielding CYP157C1WT:pET17b) and transformed into *Escherichia coli* strain BL21(pLys)DE3 for expression analysis. The QuikChange mutagenesis kit (Stratagene) was used for the construction of site-directed mutants employing PCR. The primers used for the introduction of mutations were as follows: L300R forward: 5'-ATGACGGTGCCCCAGGCGGTCTGAGCAGTCCCGTTGGGACGAGCCG-3'; L300R reverse: 5'-CGGCTCGTCCCAACGGGA-CTGCTCGACCGCTGGGGCACCCTGAT-3'; Q298E forward: 5'-CAGGCGGCTCAGGAGACTCCCTGTGGGAC-3'; Q298E reverse: 5'-GTCCACAGGGACTCCTCGACCGCCTG-3'; W301R forward 5'-CGGCGGCTCGTCCCGCAGGGACTGCTC-3'; W301R reverse 5'-GAGCAGTCCCTGCGGGACGAGCCGCCG-3'. The integrity of the CYP157C1 wildtype and mutant sequences were confirmed by DNA sequencing.

2.3. Characterisation of expressed proteins

Single ampicillin/kanamycin-resistant colonies from each transformant were grown overnight at 37 °C in 5 ml of Terrific Broth consisting of 1.2% (w/v) Bactotryptone (Difco), 2.4% (w/v) yeast extract (Difco), 0.4% (v/v) glycerol supplemented with 100 µg/ml ampicillin and 50 µg/ml kanamycin. These pre-cultures were inoculated into 500 ml of modified Terrific Broth media also containing 100 µg/ml ampicillin and 50 µg/ml kanamycin (1:100 culture dilution). After 4 h growth at 37 °C in a shaking incubator at 240 rpm, the culture was induced to express CYP by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and δ-aminolevulinic acid to a final concentration of 1 mM (to ensure optimal CYP expression) and induced to express chaperones by addition of arabinose to a final concentration of 4 mg/ml. Using three 500 ml cultures (1.5 l total), growth was continued at 28 °C with shaking at 190 rpm for 38 h and the *E. coli* cells were pelleted by centrifugation at 1500 × g and resuspended in 100 ml potassium phosphate buffer (pH 7.4) containing 500 mM sodium acetate, 20% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.1 mM PMSF, 1% sodium cholate and 1% Tween 20. Cells were broken by freeze-thawing as previously described [11] and the cytosolic fraction was separated from cell debris and the membrane fraction by ultracentrifugation at 100 000 × g for 45 min.

2.4. Molecular modelling of CYP157C1 and high throughput substrate docking

A molecular model of CYP157C1 was generated after aligning 13 Class I and Class III P450s to create a “fixed” alignment as described in [13]. In this alignment, CYP157C1 shared the highest amino acid identity with CYP154C1 (28.8%). Therefore, models were generated using the crystal structure of CYP154C1 [14] as a single template and MOE programs (Chemical Computing Group, Montreal). CYP157C1 model has a normalized Z score of 0.90 (obtained from ProsaII), and a normalized 3D-1D score of 0.98 (from Profiles 3D) well above the cutoff of 0.6.

3. Results and discussion

As previous heterologous expression of CYP157C1 in *E. coli* produced extremely low levels of protein (<10 nmol CYP/L culture) preventing biochemical analysis of this protein [11], we established an efficient system for expression by coexpressing it with the molecular chaperones GroES and GroEL that have been shown to enhance the production of active and correctly folded human CYPs [15,16]. In this co-expression system, GroES and GroEL coding regions are under the control of the *AraB* promoter allowing them to be induced by the addition of arabinose. In the presence of these proteins, expression of active CYP157C1 was greatly enhanced with levels of CYP reaching >250 nmol P450/L culture after 38 h of culture. Subsequent fractionations indicated that the overexpressed CYP157C1 was soluble and capable of generating

	K	β 1-5	β 2-1	β 2-1	β 1-4	K'
CYP157A1	AASLFGGRNSVAEAMNEVLWEDTPTQNV - AGRWAARDTQLGGRRIRAGDLVLLGLQGANS	321				
CYP157B1	ALNVSGGRLSVGREALNEVLWLDTPTQN F - IGRWAVNDTQLGGRRIRAGDCLVLGFAAANT	335				
CYP157C1	RAQLSGGQMTPVQAWEQSLWDEPPFSTV - FAYFAKQETELGGQRIRAGDGLLLGIAPGNV	340				
CYP157C2	RQNLGGQMTPVEAVEQSLWDEPPFSTV - LGYFAKQETELGGQLIRKGDGLLLGIAPGNV	342				
CYP157C3	RARLSGGHMTVPEAVEQTLDWDEPPFTAV - FGRWAVGDTTELGGQQIKAGDALLVGIAPANT	340				
CYP157C4	RANLAGGHMTLPDALEQVLWDDEPPLSVI - TGRWATGDTVLGKKQISKGDMLLLGLAAGNV	339				
CYP154C1	RALVLSGEAEWSAVVEETLRWFSTPTSHV - LIRFAAEDVPVGDRVIPAGDALIVSYGALGR	323				
CYP158A2	AERLRSEPIRPRIDEELRLWI PHRNAVGLSR LAIDVEV I KGVRI RAGDAVVYSYLAANR	323				
 *	*	*	* * * . .	

Fig. 2. K-helix alignment of five members of the CYP157 family with CYP154C1 and CYP158A2 from *Streptomyces coelicolor* A3(2). The alignment of *S. coelicolor* A3(2) CYP157A1, CYP157B1 and CYP157C1, *S. avermitilis* CYP157A2 and CYP157C2 and *S. coelicolor* A3(2) CYP158A2 and CYP154C1 is shown with the variant EXXR motif in the CYP157 subfamily shown in aqua and the consensus EXXR motif in CYP158A2 and CYP154C1 shown in red.

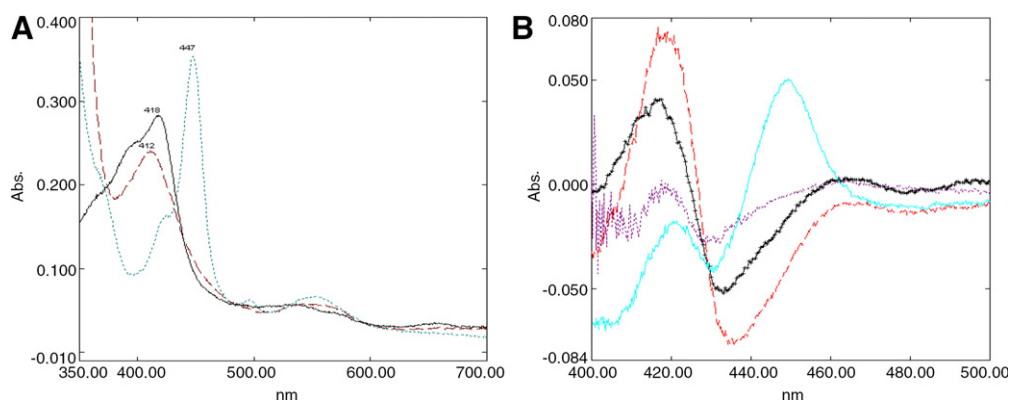


Fig. 3. Reduced carbon monoxide difference spectral analysis of wild type and mutant CYP157C1 proteins. Spectra were recorded from bacterial cytosolic extracts as described in Section 2. (A) Native CYP157C1 shows classical P450 spectral oxidized (red), reduced (black), reduced-CO (blue), (B) Reduced carbon monoxide difference spectra, wild type (aqua), Q298E mutant (purple), W301R mutant (black), Q298E/W301R double mutant (red).

a spectral maximum at 448 nm in reduced carbon monoxide difference analysis (Fig. 3).

Site-directed mutants of CYP157C1 with altered K-helix regions were expressed using this chaperone co-expression system. According to resolved crystal structures of many prokaryotic and eukaryotic CYPs, the invariant EXXR motif found in the K-helix of both classes of these P450s forms a network of salt-bridge interactions that are proposed to facilitate haem binding [8]. However, in CYP157C1 the EXXR region is absent and replaced with the Q²⁹⁸SLW³⁰¹ motif. Clearly, these substitutions are not conservative with the negatively charged glutamate being replaced with the uncharged polar amide derivative glutamine and the positively charged arginine replaced with the hydrophobic residue tryptophan.

To assess the importance of these residues in the tertiary structure of CYP157C1, the native E²⁹⁷QSLW³⁰¹ sequence was changed to E²⁹⁷ESLR³⁰¹ as well as to E²⁹⁷QSRW³⁰¹ both containing the more canonical EXXR sequence. While creation of the first of these site-directed mutants necessitated replacements at two positions within this heptapeptide, creation of the second of these site-directed mutants took advantage of the proximal E²⁹⁷ to accommodate construction of this motif. As shown in Fig. 3, reduced carbon monoxide spectra revealed mostly incorrectly folded protein for the E²⁹⁷ESLR³⁰¹ double mutant and each of its single mutants; the same results were also found for the E²⁹⁷QSRW³⁰¹ single mutant (data not shown) and for CYP157C1 mutants containing EXXR which are expressed in the absence of chaperones. These results indicate that, in this CYP, EXXR is not required for correct protein folding in direct contrast to the CYP superfamily as a whole. Further, our results reveal P420 production is not through uncoupling of protein synthesis and haem incorporation. The introduction of the double mutant shows that EXXR is not tolerated within the CYP157C1 tertiary structure as this P450 has evolved to dispense of this motif. Expression with chaperones would have helped P450 production as the results of the wild type reveal, but this was not the case for the mutant expression analysis. The several previous studies indicating that EXXR is critical for correct P450 folding include mutagenesis of Arg365 in CYP19A1 which led to the production of inactive and misfolded protein [17] and mutagenesis of the corresponding Arg residue in CYP1A2 which led to inactive protein that could not bind haem [10]. In other studies,

Hatae and colleagues [9] mutated both Glu347 and Arg350 of CYP8A1 to Ala which again abolished enzyme activity while mutation of Glu359 and Arg362 in bovine CYP17A1 also led to inactive protein (Barnes and Waterman, unpublished data). In all cases, no correctly folded P450s were observed.

To provide more insight into the reasons that CYP157C1 can correctly fold in the absence of the conserved EXXR, a molecular model was built using our resolved crystal structure of *Streptomyces coelicolor* CYP154C1 as a template [14]. Fig. 4 shows the CYP154C1 crystal structure (top panel) compared with our derived CYP157C1 model (bottom panel) with the E and R (CYP154C1) and Q and W (CYP157C1) residues highlighted. In CYP154C1, the highly conserved EXXR motif in the K-helix can form not only a salt-bridge interaction within it, but also an extensive network of hydrogen bonds with adjacent amino acid residues located in meander region. For example, the guanidium group of R²⁸⁴ forms hydrogen bonds with the carbonyl oxygen atoms of A³³² and R³³⁴. The side chain of E281 can make hydrogen bonds interaction with D³³⁶ and R³³⁹ (Fig. 4, top). In our CYP157C1 model, these hydrogen bonding contacts are disrupted owing to presence of residues Q²⁹⁸ and W³⁰¹. Although Q²⁹⁸ and W³⁰¹ cannot form hydrogen bonds with each other, the side chain of Q²⁹⁸ can still make hydrogen bonds with residues in the meander region and the adjacent D³⁰² makes extensive hydrogen bonds with the same region (Fig. 4, bottom), which may stabilise the incorporation of haem. The predicted orientation of Q²⁹⁸SLW³⁰¹ in the CYP157C1 model does not affect the helical conformation of the K-helix. Since we have made mutations in the K-helix creating an EXXR but have not made mutations in the meander region, it is possible that the synthetic EXXR motif in CYP157C1 cannot support normal P450 folding because the meander is not designed to match correct hydrogen bonding. It is not obvious what mutations might be made in the meander region to match a synthetic EXXR in CYP157C1, but future studies should address this point. However, we can conclude EXXR is not required for normal P450 folding in CYP157C1. Perhaps other hydrogen bonds predicted in our model stabilize haem binding (Fig. 4).

The biological role of CYP157C1 remains unknown but clues to function can be gained from comparison of the CYP157C1 genetic organization with other *S. coelicolor* genes

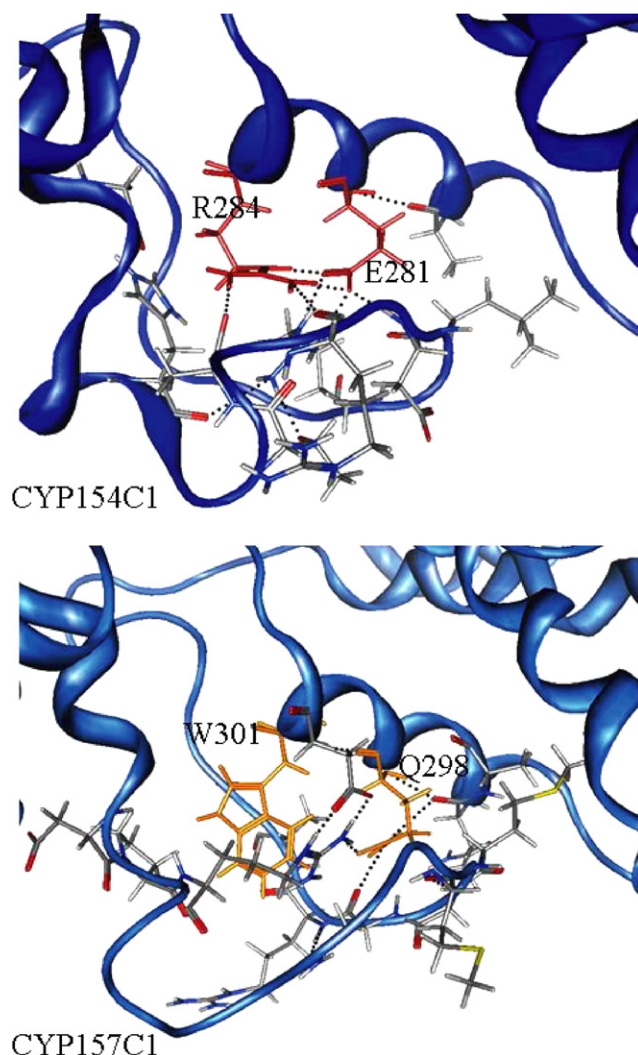


Fig. 4. Location of the EXXR and QXXW sequences in the CYP154C1 crystal structure (top) and CYP157C1 model (bottom). The presence of E and R in CYP154C1 (shown in red) can form salt-bridge interaction with each other, and also they are able to form an extensive network of hydrogen bonds with adjacent amino acid residues located in meander region, which has been speculated to allow stable incorporation of haem during correct CYP protein folding. However, the hydrogen bond network in CYP157C1 in very different because of the presence of Q and W residues (shown in orange) suggesting an as yet undetermined mechanism(s) for haem stabilization in this CYP. The potential hydrogen bonds are shown in dotted lines. The side chain atoms are rendered as stick figures (grey, carbon; blue, nitrogen; red, oxygen).

and also from codon frequencies in this gene. First, *CYP157C1* is the terminal gene of a five gene cluster termed 'conservon' [18]. In *S. coelicolor* A3(2), 13 such clusters were found (*cvnA*, *B*, *C*, *D*, *I*–*13*) with unidirectional transcription and overlaps in translational start and stop codons suggesting that these exist as a single operon [18]. Each conservon contains a predicted sensor kinase (*cvnA*), an ATP-binding protein (*cvnD*) as well as two ORFs of unknown function (*cvnB* and *cvnC*). In four of these *S. coelicolor* A3(2) conservons, the next downstream ORF codes for a CYP and, in two of these conservons, two downstream CYPs exist. In *S. coelicolor* A3(2) *cvn10*, the downstream CYPs include CYP157A1 (which has no EXXR

motif) and CYP154C1 (which has the EXXR motif); in *cvn11*, the downstream CYP is CYP157B1 (which has no EXXR motif) [11]; in *cvn12*, the downstream CYPs are CYP156A1 (which has no EXXR motif) and CYP154A1 (which has the EXXR motif); in *cvn13*, the downstream CYP is CYP157C1 (which has no EXXR motif). Analysis of the genome sequence of *S. avermitilis* has also identified 11 conservons with two of these followed by downstream ORFs coding for CYPs [19]. In *cvnC9*, the downstream CYPs are CYP157A2 (which has no EXXR motif) and CYP154C2 (which has the EXXR motif) and, in *cvnC10*, the downstream CYP is CYP157C2 (which has no EXXR motif). It is clear from these organizations that the CYP157 family proteins which lack consensus EXXR motifs are all genetically linked to their upstream conservons suggesting that they have functions linked to the encoded pathway(s). The involvement of CYPs in the function of these conservons is a key target for studies on the roles of CYP in streptomycetes and should lead to the determination of the function of CYPs lacking the conserved EXXR motif.

Second, studies in *S. coelicolor* A3(2) have indicated that mutation of *bldA* (encoding the tRNA for the rare leucine UUA codon) causes pleiotropic deficiencies in both the development of aerial hyphae and the production of antibiotics [20]. The completed *S. coelicolor* A3(2) genome identified a total of 145 genes containing one or more TTA codons and each was affected by *bldA* mutations [18]. Given that *bldA* deletion itself is not lethal to the organism then it can be assumed that mutations of any of the 145 genes containing a TTA codon are also not lethal. CYP157C1 is the only member of the P450 superfamily in *S. coelicolor* containing a rare TTA codon. Hence, it can be speculated that CYP157C1 has a role in the production of aerial hyphae or, more likely, a role in antibiotic production via oxidative tailoring associated with *bldA*. Gene deletion of CYP157C1 followed by comparison of secondary metabolite compositions in wild type and mutant strains is currently being investigated as a means to deciphering the biological function of CYP157C1.

Lastly, one other *S. coelicolor* A3(2) P450 (CYP156A1) does not contain an EXXR motif [10] but, like other CYP157 family members not containing EXXR, it is expected to be a functional enzyme. It certainly will be interesting to solve the high resolution X-ray structure of one of these CYPs to obtain a detailed understanding of how the absence of EXXR influences P450 structure. Even after completion of such structures, however, the evolutionary description of why certain CYPs have eliminated EXXR from their sequence will remain puzzling.

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